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Crystal structure of the Habc domain of neuronal syntaxin from the squid *Loligo pealei* reveals conformational plasticity at its C-terminus

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Abstract

Background: Intracellular membrane fusion processes are mediated by the spatial and temporal control of SNARE complex assembly that results in the formation of a four-helical bundle, composed of one vesicle SNARE and three target membrane SNARE polypeptide chains. Syntaxins are essential t-SNAREs and are characterized by an N-terminal Habc domain, a flexible linker region, a coiled-coil or SNARE motif and a membrane anchor. The N-terminal Habc domain fulfills important regulatory functions while the coiled-coil motif, present in all SNAREs, is sufficient for SNARE complex formation, which is thought to drive membrane fusion.

Results: Here we report the crystal structure of the Habc domain of neuronal syntaxin from the squid *Loligo pealei*, s-syntaxin. Squid Habc crystallizes as a dimer and the monomer structure consists of a three-helical bundle. One molecule is strikingly similar to mammalian syntaxin IA while the second one shows a structural deviation from the common fold in that the C-terminal part of helix C unwinds and adopts an extended conformation.

Conclusion: Conservation of surface residues indicates that the cytosolic part of s-syntaxin can adopt an auto-inhibitory closed conformation that may bind squid neuronal Sec1, s-Sec1, in the same manner as observed in structure of the rat nSec1/syntaxin IA complex. Furthermore, despite the overall structural similarity, the observed changes at the C-terminus of one molecule indicate structural plasticity in neuronal syntaxin. Implications of the structural conservation and the changes are discussed with respect to potential Habc domain binding partners such as Munc13, which facilitates the transition from the closed to the open conformation.

Background

SNARE (soluble NSF attachment protein receptor) proteins are an evolutionary conserved family of membrane-anchored or membrane-associated proteins, which contain a conserved 60 amino acid motif of hydrophobic heptad repeats typical for coiled-coil interactions [1]. Complexes formed from cognate SNARE motifs located

on opposing membranes physically link membranes prior to membrane fusion. Specifically, three SNARE chains from the target membrane and one chain from the vesicle align in a parallel four-helical bundle [2]. In neurotransmission, the vesicle SNARE synaptobrevin (also VAMP) forms a highly stable complex with the synaptic membrane proteins syntaxin 1A and SNAP-25, which contains

two SNARE motifs [3,4]. Deletion or ablation of any of these proteins resulted in a complete block in Ca^{2+} -evoked neurotransmission [5-8]. Although trans-SNARE complex formation is necessary and sufficient for *in vitro* vesicle fusion [9,10], studies with neurotoxins like Botulinum and Tetanus toxin, which block neurotransmission by proteolytically cleaving SNARE proteins that are not engaged in SNARE complex formation, indicate that SNARE complex formation occurs upstream of regulated neurotransmitter release *in vivo* [11].

While synaptobrevin and SNAP-25 comprise little more than SNARE motifs and a membrane anchor, syntaxin 1A contains an additional N-terminal domain, which was implicated in regulation of SNARE complex assembly [12-14]. The Habc domain from syntaxin, also classified as a Qa-SNARE [15,16], folds into a three-helical bundle [17,18]. Such a conserved, autonomously folded domain is characteristic of all Qa-SNAREs and is found in some Qb SNAREs such as Vti1b [19-22]. In the neuronal SNARE complex the Habc domain is connected to the SNARE motif by a highly flexible linker [23].

When not engaged in SNARE complex formation, the linker region and most of the SNARE motif in rat syntaxin 1A can fold back onto the Habc domain and form a "closed conformation", that interacts with nSec1 (also Munc18-1), an essential positive regulator of neurotransmission [24-26]. nSec1 belongs to the evolutionary conserved family of SM proteins (named after the yeast homologue Sec1p and the mammalian counterpart Munc18), which are involved in all eukaryotic membrane fusion processes [27]. Removal of the Habc domain or mutations resulting in an open conformation significantly accelerate SNARE complex formation *in vitro* [12-14]. This indicated that nSec1 acts as a negative regulator of membrane fusion. Further evidence however, suggests that nSec1 also plays a positive regulatory role in SNARE-mediated membrane fusion [28-30]. In addition, the pre-synaptic active zone proteins Munc13-1 and -2 that are essential for priming of synaptic vesicles [31-34] interact with the Habc domain of syntaxin 1 [35]. In order to further investigate the evolutionary conservation of the N-terminal domains of neuronal syntaxin between molluscs and mammals, we determined the crystal structure of the Habc domain derived from the neuronal syntaxin, s-syntaxin, of the squid *Loligo pealei*.

Results and discussion

s-Syntaxin characterization

s-Syntaxin (residues 2 to 175, additional N-terminal sequence: MRGSPR) comprising the N-terminal Habc domain and the linker region was produced in *E. coli* and originally tested whether it is sufficient for interaction with s-Sec1, different from the analogous closed nSec1/

syntaxin 1A conformation [25]. However, no interaction between s-Sec1 and s-syntaxin(2-175) could be detected by conventional methods including affinity chromatography pull-down, co-purification and native gel electrophoresis (data not shown). Given the fact that nSec1 plays a positive regulatory role during membrane fusion [27], we speculated that a potential low-affinity binding might be sufficient for its action as part of a postulated multi-protein complex at the site of fusion. We therefore tried to co-crystallize s-Sec1 and s-syntaxin in order to trap such potential low-affinity interactions, which however resulted only in crystals containing s-syntaxin. Recently, it has been shown that other SM proteins such as Sly1p and Vps45p interact with an N-terminal helical peptide of their cognate syntaxins mediated by contacts with domain I of the respective SM protein different from the nSec1 closed conformation syntaxin 1A interaction [36-39]. Although s-syntaxin contains this potential peptide region, which has been predicted to have a propensity for α -helix formation [22], no binding *in vitro* and no co-crystallization could be detected. We can, however, not exclude the possibility that the extra sequences at the N-terminus of s-syntaxin (MRGSPR) might have interfered with binding to s-Sec1.

Structural overview

The crystals of s-syntaxin belong to space group $P4_32_12$ and diffracted X-rays to 3.3 Å resolution using synchrotron radiation. The asymmetric unit contains two molecules of s-syntaxin (molecules A and B, Figure 1A), which both fold into three-helical bundle structures with an approximate length of 45 Å. By convention, the helices are named Ha, Hb and Hc from N- to C-terminus. The N-termini, which include the proposed helical segment, the C-terminal linker to the SNARE motif and all but one of the loop regions connecting the helices were disordered in both molecules. The final models start at residues 31 or 32 and their C-terminal ends are defined by residues 151 (molecule A) and 156 (molecule B). $\text{C}\alpha$ -Atoms of residues 32 to 142 from the two molecules can be superposed with an r.m.s. (root mean square) deviation of 0.555 Å. However, the C-terminal ends diverge substantially (Figure 1B). In molecule A, the third helix Hc is almost four helical turns shorter and the corresponding part, residues 143 to 151, which is highly conserved among syntaxins (Figure 3) forms an open coil structure which binds to a hydrophobic cleft opening between helices Ha and Hc of molecule B (Figure 1A). Although, the C-terminal coil region in molecule A (residues 143 to 151) and the corresponding helical region in molecule B have elevated B-factors compared to the overall model (A, 67.0 Å², and B, 66.1 Å², versus all atoms, 42.3 Å²), the electron density in this region is well interpretable (Figure 2). The extended conformation is stabilized by an intermolecular disulfide bond, Cys148A-Cys148B, and three hydrogen bonds (Fig-

ure 2). It should be noted, that if both syntaxin molecules assumed a conformation like in molecule B severe steric clashes would result. In molecule B, Cys148 lies rather protected at the interface of helices Hb and Hc. This indicates that the C-terminal end of the Habc domain can adopt different conformations, which may be important when interacting with different regulatory partners thus adding to the structural plasticity observed for neuronal syntaxins [40]. In accordance, a recent time-resolved FRET (fluorescence resonance energy transfer) study indicates that rat neuronal syntaxin 1A undergoes fast conformational conversion with only a minority population in the closed conformation [41]. Structural flexibility at or close to the linker region of syntaxin might promote rapid inter-conversion of conformations. Therefore, this additional syntaxin conformation might be of physiological relevance as it is not obvious how local unfolding would have been favored by the mild crystallization conditions (see Methods).

Molecule B and the core of molecule A are however strikingly similar to mammalian syntaxin 1A. The secondary structure of the three-helical bundle domain of rat neuronal syntaxin was initially determined by NMR (pdb code 1BR0 [17]) and confirmed by X-ray crystallography (pdb code 1EZ3 [18]). In addition, the linker region and the SNARE motif of neuronal syntaxin fold back onto the three-helical bundle in the closed conformation, as observed in the crystal structure of the complex with nSec1 (pdb code 1DN1 [25]), which leaves the N-terminal domain virtually unaltered.

The C α atoms of squid (molecule B) and rat neuronal syntaxin (X-ray structure, chains A to C [18]) can be superposed with r.m.s. deviations between 0.711 and 0.761 Å for residues 32 to 149 (not shown). These values are very close to the deviation between the two copies in the asymmetric unit of squid syntaxin itself and therefore the structures of rat and squid neuronal syntaxin can be considered virtually identical despite the considerable evolutionary distance. The deviation from the all-helical fold at the C-terminal region of the Habc domain in molecule A, however, is novel albeit its functional significance has yet to be proven.

Sequence conservation and functional implications

Within animals, neuronal syntaxin is remarkably well conserved. The homologues of rat, *H. medicinalis*, *C. elegans* and *D. melanogaster* share 47.5 % sequence identity (80.3 % homology) with the sequence of the squid *L. pealei* (Figure 3). In comparison to the SNARE motif, which is highly conserved (residues 183 to 255, 63 % identity, 86.3 % homology), the three-helical bundle domains share somewhat lower sequence identity (residues 32 to 154, 50.8 % identity, 75.6 % homology). The

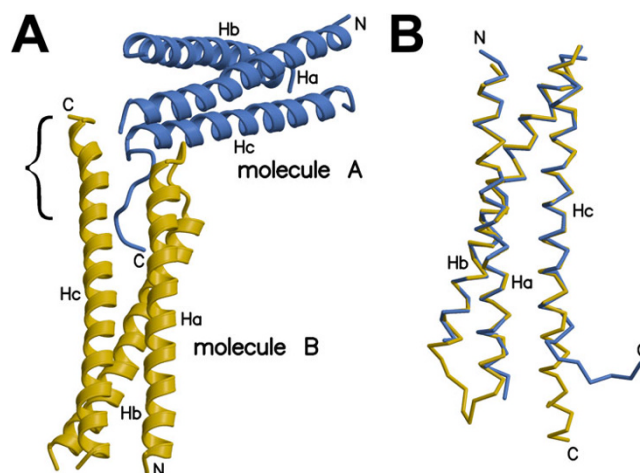


Figure 1
Asymmetric unit content in crystals of neuronal syntaxin from *L. pealei* (A) Ribbon diagram of the dimer in the asymmetric unit. N- and C-terminal ends are indicated. A brace indicates the C-terminal Habc region (in molecule B), which can have either helical or extended conformation. (B) Overlay of the C α backbone of molecules A and B. Note that the C-terminus of molecule A deviates from the helical fold of Hc from molecule B as 4 helical turns are unwound.

surface of the Habc domain is highly conserved however (Figures 4B and 4E), underlining their functional conservation. While the N-terminal region preceding the three-helical bundle is generally the least conserved region in neuronal syntaxin, the ultimate N-terminus, which comprises the SM protein binding signature region, residues 1 to 18, in the syntaxin homologues Sed5p and Tlg2p exhibits remarkable conservation [36-38]. This is even more pronounced among the invertebrate sequences. However, *in vitro* binding of this region of neuronal syntaxin to nSec1 could not be shown experimentally for both the rat and the squid proteins [39,42] (unpublished observations).

Surface regions of high amino acid sequence conservation within neuronal syntaxins match very well with the sites of intramolecular interactions in the closed conformation. Indeed all residues involved in the formation of the closed conformation as well as those involved in contacting nSec1 in the complex structure [25] are strictly conserved between squid and rat syntaxin (Figures 3, 4). Therefore, we predict that squid neuronal syntaxin can assume the same closed conformation as its rat homologue and that it will bind s-Sec1 in the same way [16], although the degree of conservation between squid and rat neuronal SM proteins is somewhat lower (66 % vs. 83 % identity).

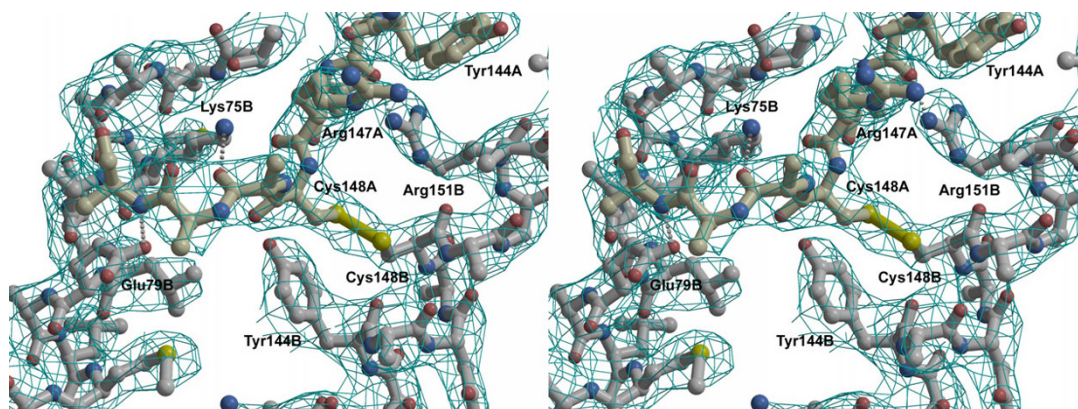


Figure 2

Stereo diagram of the electron density at the interface of the s-syntaxin dimer. $2F_o - F_c$ electron density at 1.0σ of the contact region between molecule B and the C-terminus of molecule A is shown as a meshwork in cyan. Carbon atoms in molecules A and B are shown in beige and grey, respectively. Heteroatoms are indicated in red (oxygen), blue (nitrogen) and yellow (sulphur). Dashed lines indicate polar interactions stabilizing such a conformation. Residues 32 to 69 in molecule B have been omitted for clarity.

The syntaxin binding partner Munc13 (murine Unc-13) was found to be essential for neurotransmitter release in organisms ranging from *C. elegans* to mammals [31,32,43], a conservation which also postulates a squid homologue of Munc13. It has been proposed that Munc13 facilitates the transition from the closed syntaxin conformation in complex with nSec1 to the open state participating in SNARE complex assembly [43,44]. This is in accordance with biochemical data showing that Munc13 interacts with the three-helical bundle domain of neuronal syntaxin in a yeast two-hybrid screen [35]. Specifically, the binding region was located between residues 53 and 79 in rat neuronal syntaxin, which includes part of Ha, the linker and part of Hb (Figure 3). The proposed binding site partly overlaps with the binding of the SNARE motif in the closed conformation, which might render nSec1 and Munc13 interactions exclusive.

Interestingly, the surface corresponding to this region also contains highly conserved residues, which are involved in neither the intramolecular closed conformation nor the intermolecular nSec1 interactions (arrow, Figure 4). In addition, the extended conformation of the C-terminal end of Habc locates next to this putative Munc13 interaction site. Therefore it is possible that the structural changes might be either necessary for Munc13 binding or might be a consequence of Munc13 binding or of some other potential ligand *in vivo*.

Another possible interaction partner of syntaxin that is capable of dissociating Munc18 from syntaxin-1 is tomosyn [45], which might imply interaction with the Habc domain. On the other hand, a C-terminal SNARE motif in tomosyn competes for SNARE complex assembly [46].

Function of the three-helical bundle domain of neuronal syntaxin

Three-helical bundle Habc domains appear to be a general feature of syntaxin-type SNARE proteins, e.g. the paralogous yeast syntaxins Sso1p, Sed5p, Tlg2p and Vam3p have all been shown to contain homologous domains [20,21,36,37]. Furthermore, Q-SNARES such as syntaxin 6, syntaxin 7 and Vti1p, which substitute SNAP-25 in the endosomal SNARE complex, contain structurally similar three-helical bundle domains [19,22].

The Habc domain of neuronal syntaxin interferes with SNARE complex formation by promoting the closed conformation and serves as a scaffold for the interaction with nSec1. However, only the syntaxin homologue Sso1p was shown to form a stable closed conformation [21]. Sec1p, the corresponding SM protein and the closest homologue to nSec1 in yeast, binds exclusively to the assembled SNARE complex [47]. Furthermore, short N-terminal signature sequences in the yeast syntaxin homologues Sed5p and Tlg2p and their mammalian counterparts are sufficient for recruitment of the respective SM proteins Sly1p and Vps45p [36-38].

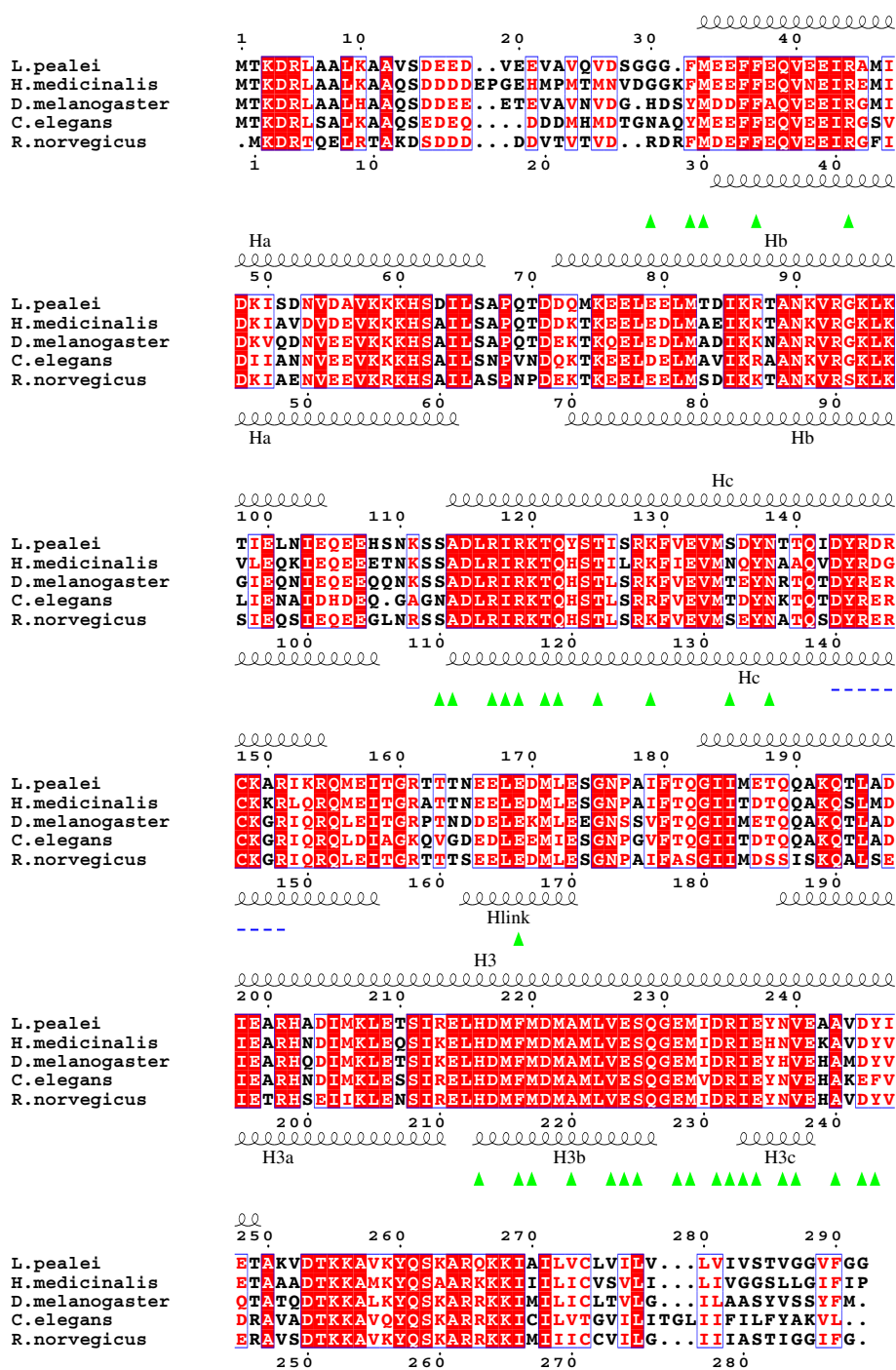


Figure 3
Structure based sequence alignment of neuronal syntaxins Sequences from five animal phyla (SwissProt/ TrEMBL accession codes, *L. pealei* O46345, *H. medicinalis* O01390, *D. melanogaster* Q24547, *C. elegans* O16000, *R. norvegicus* P32851) were used. Secondary structure elements are indicated for neuronal syntaxin from *L. pealei*, above the sequence, based on the crystal structure of the Habc domain and the squid SNARE complex (pdb code 1L4A) [62]. Secondary structure element assignment for the rat homologue is based on the X-ray structure of syntaxin 1A in complex with nSec1 (pdb code 1DN1) [25] and is shown below the sequence, thus highlighting the different conformations that can be adopted by the SNARE motif. Identical residues are indicated by red background. Homologous residues are shown in red. Green triangles indicate syntaxin residues that interact with nSec1 in the closed conformation [25]. The region with divergent conformations at the C-terminus of the squid Habc domain is marked with a blue dashed line.

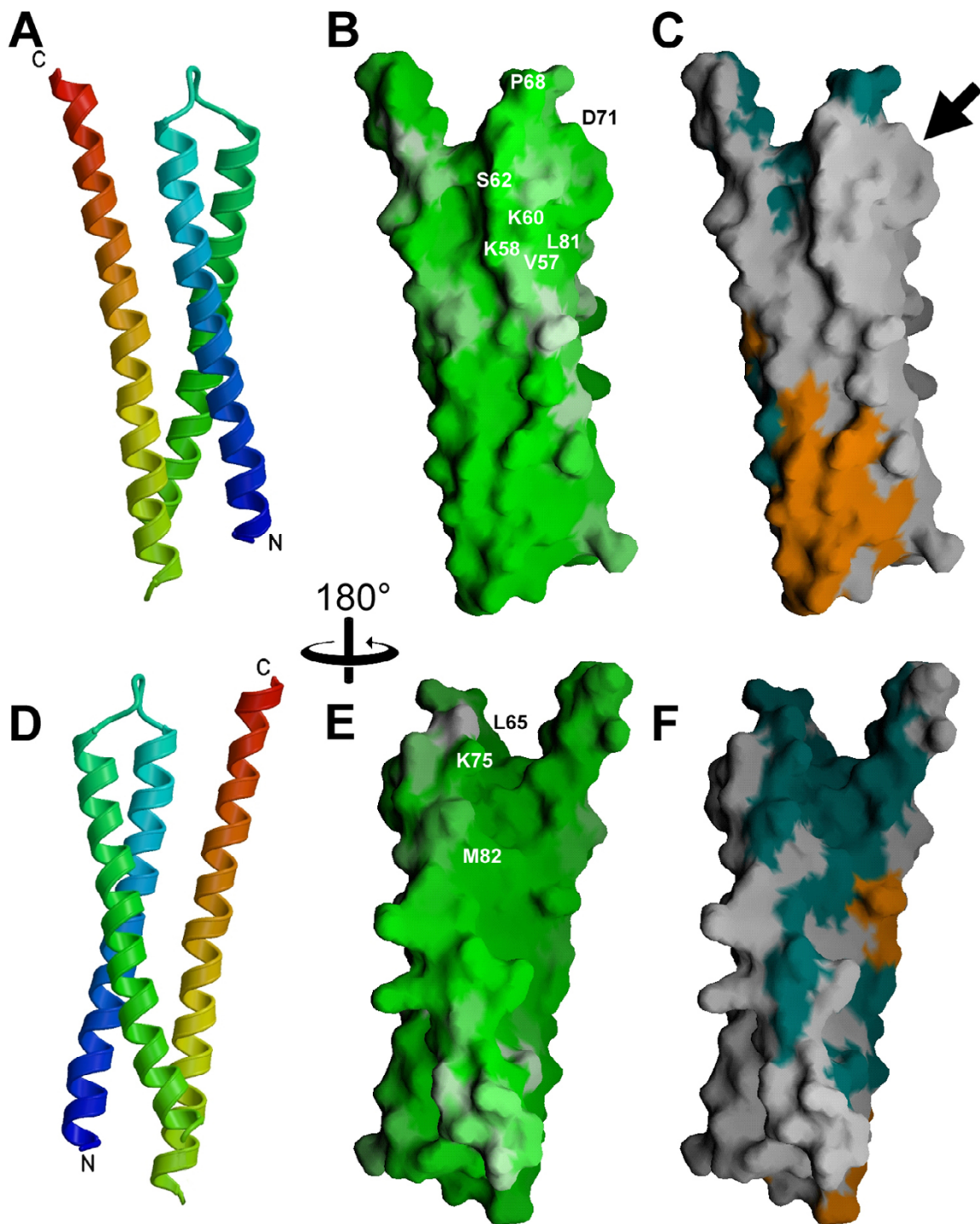


Figure 4

Surface conservation of squid neuronal syntaxin Two projections of squid neuronal syntaxin related by an 180° rotation are shown. In the middle panels B and E, shades of green indicate the degree of amino acid conservation within distantly related neuronal syntaxins, white, no conservation, and dark green, absolute conservation. The surface conservation score was calculated with ESPrpt based on the sequence alignment in Figure 3[60]. Conserved surface residues in the putative Munc13 binding region are labeled. On the right (panels C and F), rat syntaxin IA residues involved in intramolecular closed conformation and nSecI interactions are shown for comparison in turquoise and orange, respectively. Ribbon representations of syntaxin using rainbow color-coding are shown on the left for orientation (panels A and D).

Table 1: Data collection and refinement statistics

Data collection	
Data set	ESRF ID29
Wavelength (Å)	1.0088
Resolution (Å)	15.0–3.3 (3.42–3.3)
Completeness (%)	99.4 (100.0)
Total reflections	59756
Unique reflections	9304
R_{merge}	0.074 (0.375)
$\langle I \rangle / \langle \sigma \rangle$	14.8 (4.6)
Space group	$P4_32_12$
Lattice parameters (Å)	a, b = 73.056; c = 224.434
Molecules / a.u.	2
Matthews coefficient	3.74
Solvent content (%)	66.9
Refinement	
Resolution range (Å)	14.97 – 3.34
Number of reflections / test set	9251 / 500
$2R_{\text{factor}}$	0.3088
R_{free}	0.3754
Residues	227 (out of 360)
Number of protein atoms	1622
Average B factor (Å ²)	42.34
Model geometry	
Bond length r.m.s.d from ideal (Å)	0.016
Bond angle r.m.s.d. from ideal (°)	1.610
Ramachandran plot³	
% in most favoured regions	81.8
% in additionally allowed regions	16.8
% in generously allowed regions	1.4

Values in parenthesis are for last shell limits. $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I_i(\text{hkl}) - \langle I(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I_i(\text{hkl})}$ $2R_{\text{factor}} = \frac{\sum_{\text{hkl}} ||F_{\text{obs}}| - k |F_{\text{calc}}||}{\sum_{\text{hkl}} |F_{\text{obs}}|}$ ³As defined in PROCHECK [56]

The structural conservation of the autonomously folded N-terminal domain of Qa SNAREs suggest that the Habc domains might recruit not only SM proteins but also other regulatory proteins involved in vesicle trafficking. Accordingly, constitutively open full-length but not N-terminally truncated yeast Sso1p can complement a $\Delta\text{SSO1} \Delta\text{SSO2}$ yeast strain [21,48]. In addition, the formation of a closed conformation as observed in the neuronal system seems to be rather an exception among diverse syntaxins and might be necessary to ensure a tighter control of the syntaxin conformation and thus SNARE complex assembly than in other vesicle transport and fusion systems.

Conclusions

Structural and evolutionary conservation of the Habc domain of squid neuronal syntaxin indicates the fundamental role of neuronal syntaxin for neurotransmission. Almost identical conformation and strong conservation of surface residues suggest that the interplay of neuronal syntaxin with its interaction partners nSec1 and Munc13 is conserved from mollusks to mammals. Therefore, squid neuronal syntaxin undergoes the same conformational

cycle as its rat homologue. Interestingly, the structure also indicates some newly identified structural changes at the C-terminus of helix Hc, which might play an important role in the control of the conformational flexibility of syntaxins and thus in membrane fusion at the synapse.

Methods

Cloning, expression and purification

A fragment of *Loligo pealei* syntaxin comprising residues 2 to 175 was cloned into a modified pQE30 vector (Qiagen) missing the region coding for a N-terminal His6-tag (additional N-terminal sequence, MRGSPR). The protein was expressed in *E. coli* BL21 pUBS cells.

After 3 h of induction at 37°C, the cells were lysed by ultrasonication in 50 mM HEPES KOH pH 7.4. The clarified supernatant was applied to a Sepharose Q column (Amersham Biotech) at 4°C and eluted with a linear gradient to 500 mM KCl. Subsequently, 4 M ammonium sulphate was added until the pooled fractions had a final concentration of 0.8 M. The solution was applied to a Phenyl Sepharose 6 FF column (Amersham Biotech), and the unbound fraction collected. Size exclusion chromatography on Superdex200 (Amersham Biotech) equilibrated with 20 mM HEPES KOH pH 7.4 and 100 mM KCl served as final purification step.

Crystallisation

Large octahedral crystals were obtained by the hanging drop method after mixing equal volumes of 26 mg/ml s-syntaxin with reservoir buffer containing either 30 % PEG-400, 0.1 M Tris HCl pH 8.5 and 0.2 M Na-citrate or 18 – 20 % PEG-550MME, 0.1 M Tris HCl pH 8.5 and 0.2 M Na-citrate. For cryo-protection, the crystals were incubated with 30 % PEG-400, 10 % glycerol, 0.1 M Tris HCl pH 8.5 and 0.2 M Na-citrate over night. The crystals of space group $P4_32_12$ with lattice dimensions of a, b = 73.056 Å and c = 224.434 Å diffracted to 3.3 Å resolution at ESRF beamline ID29.

Structure solution and refinement

Diffraction data were processed and scaled with Denzo and Scalepack [49]. The structure was solved by molecular replacement using the program AMoRe with rat syntaxin (chain A in pdb entry 1ez3 [18]) as a search model [50]. The asymmetric unit contains two molecules. A Matthews coefficient of 3.74 Å³ Da⁻¹ and a solvent content of 66.9 % were calculated [51]. The model was refined using simulated annealing, gradient minimization and individual B-factor refinement as implemented in CNS alternated by rebuilding cycles using the program O [52,53]. In a final step, the tensor elements describing the anisotropic displacement of the individual helices of syntaxin were refined using the TLS option in Refmac5 followed by 5

cycles of restrained minimisation [54,55]. The final R-factor is 0.309 with a Rfree of 0.375.

The final model contains only 227 out of 360 residues including residues 31 to 66, 74 to 107 and 113 to 151 of chain A and residues 32 to 105 and 113 to 156 of chain B. All of the residues are in allowed regions of the Ramachandran plot as defined in Procheck [56]. Due to missing electron density for side chains, 66 solvent exposed residues were modeled as alanine, which, together with the disordered regions (> 1/3 of the protein crystal content) contribute to the somewhat high R factors.

The model coordinates were deposited at the pdb database under accession code 1s94.

Figures were generated with the programs Molscript [57], Bobscript [58], Raster-3D [59], ESPript [60] and Grasp [61].

Authors' contributions

A.B. carried out the cloning, purification, crystallization, data collection, structure solution and refinement of syntaxin(2–175). W.W. conceived of the study, and participated in its design, coordination and writing of the manuscript. All authors read and approved the final manuscript.

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